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Antisense Downregulation of a Mouse Mammary Tumor Virus Activated Protooncogene in Mouse Mammary Tumor Cells Reverses the Malignant Phenotype

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Activation of the protooncogene *Wnt*-1 by insertion of the mouse mammary tumor virus (MMTV) is known to cause mammary tumors in mice. *Wnt*-1 expression in mammary glands has been postulated to confer direct local growth stimulation of mammary epithelial cells leading to their acquisition of a preneoplastic state. *Wnt*-1 expression also induces morphological alterations in cultured normal mammary cells. However, it has not been determined whether or not transformed mammary cells require continuous *Wnt*-1 expression for their ability to form tumors *in vivo*. To address this question, we constructed antisense and sense *Wnt*-1 expression vectors containing a synthetic promoter composed of five high-affinity glucocorticoid response elements (GRE5). This promoter is at least 50-fold more inducible by dexamethasone than the promoter contained in the long terminal repeats of MMTV. The vectors were introduced into a mouse mammary tumor cell line (R/Sa-MT) that expresses high levels of endogenous *Wnt*-1 mRNA and forms rapidly growing tumors when transplanted into syngeneic hosts. Of the 12 stably transfected cell lines established (9 with antisense and 3 with sense constructs), 2 antisense cell lines (R/Sa-MT/antisense) and 1 sense cell line (R/Sa-MT/sense) were examined for inducibility by dexamethasone of antisense and sense *Wnt*-1 RNAs, changes in endogenous *Wnt*-1 RNA expression, and changes in cell morphology. The growth patterns of the cells *in vitro* and *in vivo* were also examined. Our results show that (1) the levels of the expression of endogenous *Wnt*-1 mRNA and protein were reduced significantly (>80%) in those cells (R/Sa-MT/antisense) that expressed antisense *Wnt*-1 RNA at high levels following exposure to dexamethasone, compared to the R/Sa-MT/sense and R/Sa-MT control cells and (2) transplantation of the R/Sa-MT/antisense cells produced smaller tumors (≈0.2 cm in 16 weeks) compared to the tumors (≈2.0 cm in 8 weeks) that were produced by the R/Sa-MT/sense and R/Sa-MT cells. We therefore suggest that *Wnt*-1 expression is required not only for the transformation of normal mammary cells into tumor cells, but also for the maintenance of their tumorigenicity.

Key Words: Wnt-1 protooncogene; antisense RNA; cell morphology; mouse mammary tumor; tumor growth.

INTRODUCTION

Insertional mutations caused by the mouse mammary tumor virus (MMTV) in a number of protooncogenes, such as *int*-1/*Wnt*-1 (Nusse and Varmus, 1982; Nusse *et al.,* 1984), *int*-2/*Fgf*-3 (Peters *et al.,* 1983; Dickson *et al.,* 1984; Shackleford *et al.,* 1993a), and *int*-3 (Gallahan and Callahan, 1987; Sarkar *et al.,* 1994), are thought to be involved in the development of spontaneous mammary tumors in mice. To address the question of how MMTV activated *int* genes contribute to the transformation of normal mammary epithelial cells into cancer cells, a number of structural and functional studies of the *Wnt*-1 gene have been carried out. Since its identification in 1984, *Wnt*-1 has been demonstrated to be a member of a large group of developmentally regulated genes, including the segment polarity gene *wingless* of *Drosophila* (for review see Nusse and Varmus, 1992). In fact, the *Drosophila* gene, like mouse *Wnt*-1, is capable of causing transformation and mitogenesis of mouse mammary cells and of converting them to a tumorigenic phenotype (Ramakrishna and Brown, 1993). *Wnt*-1 also normally functions in the embryonic central nervous system (Nusse and Varmus, 1992; McMahon, 1992); its expression is required for the development of a large region of the mouse brain (McMahon and Bradley, 1990). Furthermore, *Wnt*-1 expression in *Xenopus* embryos has been found to promote the duplication of the embryonic axis (McMahon and Moon, 1989).

In addition to its roles in embryonic development and mouse mammary tumorigenesis, *Wnt*-1 shows other biological activities *in vitro*. *Wnt*-1 expression has been found to induce morphological alterations in one mammary epithelial cell line (Brown *et al.,* 1986) while it rendered another cell line (RAC311C) tumorigenic (Rijsewijk *et al.,* 1987). Further, *Wnt*-1-expressing rat or mouse fibroblasts that show no detectable response themselves can transform neighboring C57BL cells in coculture experiments. This indicates that the Wnt-1 protein, a cysteine-rich glycoprotein of 41 to 44 kDa, apparently acts via a paracrine mechanism (Jue *et al.,* 1992). It is of interest to note that Wnt-1 does not normally accu-

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mulate in the tissue culture medium, but instead, is secreted onto the surface of cells or into their microenvironment (Papkoff *et al.,* 1987; Bradley and Brown, 1990; Papkoff and Schryver, 1990) and seems to be active when it exists as a transmembrane protein (Parkin *et al.,* 1993).

Results from one *in vivo* study in a *Wnt*-1 transgenic mouse model have indicated that *Wnt*-1 expression in mammary glands confers a direct local growth stimulation of mammary epithelial cells, leading to their acquisition of a preneoplastic state (Lin *et al.,* 1992). This is consistent with the finding that the mammary glands of young virgin transgenic mice (approximately 3 months old) resemble the mammary glands of pregnant nontransgenic animals with regard to the development of alveolar hyperplasias (Tsukamoto *et al.,* 1988). Even the mammary glands of 2-week-old *Wnt*-1 mice show alveolar hyperplasias (Lin *et al.,* 1992). However, it is still unknown whether these hyperplastic cells need to be continuously stimulated by the Wnt-1 protein in order for the cells to be transformed into tumor cells. More importantly, it remains to be established whether or not mammary tumor cells acquire growth autonomy during transformation or whether they still require continuous expression of *Wnt*-1 for their ability to form tumors *in vivo* and subsequently metastasize. These concerns can be addressed by reducing the expression of *Wnt*-1 in preneoplastic and/or neoplastic mammary tumor cell lines through antisense technology and testing whether the ability of the cells to form tumors *in vivo* is impaired.

The antisense approach has been successfully applied by numerous investigators to inhibit the expression of a variety of target genes, and hence, the activity of the protein product (for review see Stein and Cheng, 1993). For example, the expression of antisense RNA to the insulin-like growth factor-1 receptor has been shown to induce regression of wild-type rat glioblastoma tumors (Resnicoff *et al.,* 1994), loss of the metastatic phenotype of the Lewis lung carcinoma line H-59 (Long *et al.,* 1995), and suppression of malignant phenotypes of a human alveolar rhabdosarcoma (Shapiro *et al.,* 1994). Downregulation of N-*myc1* by antisense RNA in woodchuck hepatoma cells has recently been observed to reverse the malignant phenotype (Wang *et al.,* 1997). In the present study, we have used an antisense approach to determine the role of *Wnt*-1 in the growth *in vivo* of a mammary tumor cell line (R/Sa-MT) expressing *Wnt*-1. R/Sa-MT cells were transfected with vectors designed to constitutively express a segment of *Wnt*-1 RNA in the antisense or sense (as control) orientations relative to a steroid-inducible promoter. Stable transfectants were isolated and tested for tumorigenicity. Our results show that the expression of ectopic antisense, but not sense, *Wnt*-1-specific RNA in R/Sa-MT cells resulted in lowering significantly the levels of expression of endogenous *Wnt*-1 mRNA and protein. In addition, the cells lost their ability to form tumors in mice by more than 90%. Thus, we suggest that continuous signaling by Wnt-1 protein is required for the maintenance of the tumorigenic phenotype of mammary tumor cells in mice.

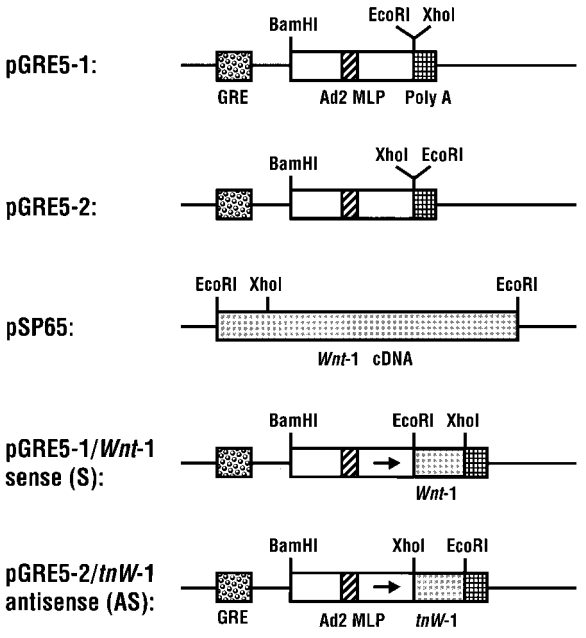

FIG. 1. Schematic representation of the construction of sense and antisense *Wnt*-1 expression plasmids. An *Eco*RI/*Xho*I fragment containing the 5′ end of the *Wnt*-1 cDNA was isolated from the plasmid pSP65 (third panel) and ligated to the expression vectors pGRE 5-1 (first panel) and pGRE 5-2 (second panel) in the sense (fourth panel) and antisense orientations (fifth panel) at the *Eco*RI/*Xho*I and *Xho*I/*Eco*RI sites, respectively. The expression vectors pGRE 5-1/5-2 contained a synthetic promoter composed of five sites for the binding of glucocorticoid receptor (GRE) and the adenovirus 2 major late promoter (Ad2) and TATA box/initiation site.

RESULTS

Hormonal regulation of antisense *Wnt*-1 RNA expression and the downregulation of endogenous *Wnt*-1 mRNA

To gain insights into the regulatory role of *Wnt*-1 in the growth of mammary tumors, we transfected a mammary tumor cell line, R/Sa-MT, with two expression vectors, pGRE5-1/*Wnt*-1(sense, S) and pGRE5-2/*tnW*-1(antisense, AS) containing a 290-bp segment of the *Wnt*-1 cDNA (Fig. 1), together with pSV2neo plasmid DNA. The rationale for our choice of the vectors was that they contained a synthetic promoter composed of five high-affinity binding sites for the glucocorticoid receptor (GRE5) placed upstream of the adenovirus 2 major late promoter (Ad2MLP) TATA box/initiation site (Fig. 1). This promoter has been shown to be at least 50-fold more inducible by dexamethasone than the MMTV long terminal repeat (LTR) promoter (Mader and White, 1993). We therefore expected that it would induce high levels of *Wnt*-1 anti-

sense RNA, but not endogenous *Wnt*-1 mRNA, in transfectants exposed to small amounts of dexamethasone.

As shown in Fig. 2, digestion of both the S and the AS plasmids with *Eco*RI/*Xho*I produced a 290-bp fragment (lanes S-E/X and AS-E/X), whereas digestion with *Eco*RI/*Bam*HI generated 650- (lane S-E/B) and 940-bp (lane AS-E/B) fragments. These results indicate that the S and AS vectors contained the 290-bp *Wnt*-1 fragment in the sense and antisense orientations, respectively. Following G418 selection of the transfected cells, we isolated a total of 23 cell clones. Expansion of these clones and examination by Southern hybridization with a *Wnt*-1 cDNA probe resulted in the identification of nine cell lines (AS-3 to AS-8, AS-12, AS-13, and AS-16) carrying one to five copies of the antisense construct and three cell lines (S-3, S-4, and S-7) that contained one to three copies of the sense construct. The presence of multiple copies of an antisense construct (lane 2) and a single copy of a sense construct (lane 6) in two stably transfected cell lines, AS-13 and S-3, respectively, is shown in Fig. 2B.

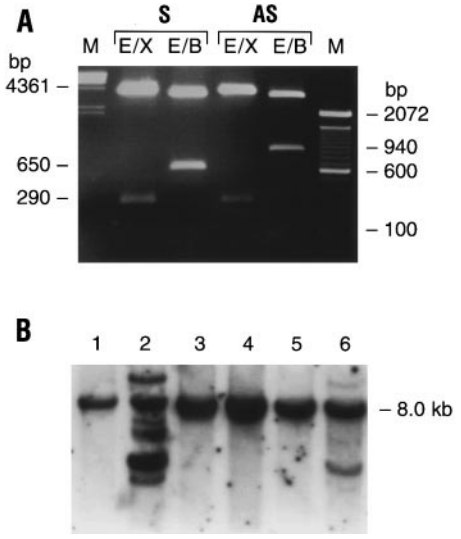

FIG. 2. (A) The pattern of restriction fragments of the plasmid constructs S and AS (see Fig. 1) that identifies the DNA clones containing *Wnt*-1 sequences in the sense (S) or in the antisense (AS) orientations. Plasmid DNA was double digested with *Eco*RI (E) and *Bam*HI (B), or *Eco*RI and *Xho*I (X), and the fragments were separated by gel electrophoresis and visualized by ethidium bromide staining. The low-molecular-weight E/X band corresponds to the 290-bp *Wnt*-1 fragment (Fig. 1) that was used to construct the sense and antisense plasmids. The presence of 650- and 940-bp E/B bands in the corresponding plasmids indicate that they contain the 290-bp *Wnt*-1 fragment in the sense and antisense orientations, respectively. (B) Southern blot hybridization with the *Wnt*-1 cDNA probe of the *Eco*RI digests of DNA for the identification of the R/Sa-MT cell clones that contained *Wnt*-1 antisense or sense constructs. Note that only one (lane 2) of the three antisense clones (lanes 2–4) and one (lane 6) of the two sense (lanes 5 and 6) clones shown in this figure contained copies of exogenous Wnt-1 sequences. The 8.0-kb band corresponds to the endogenous *Eco*RI/*Wnt*-1 fragment. Lane 1 contained control R/Sa-MT cell DNA. Lane 2, clone AS-13; lane 6, clone S-3.

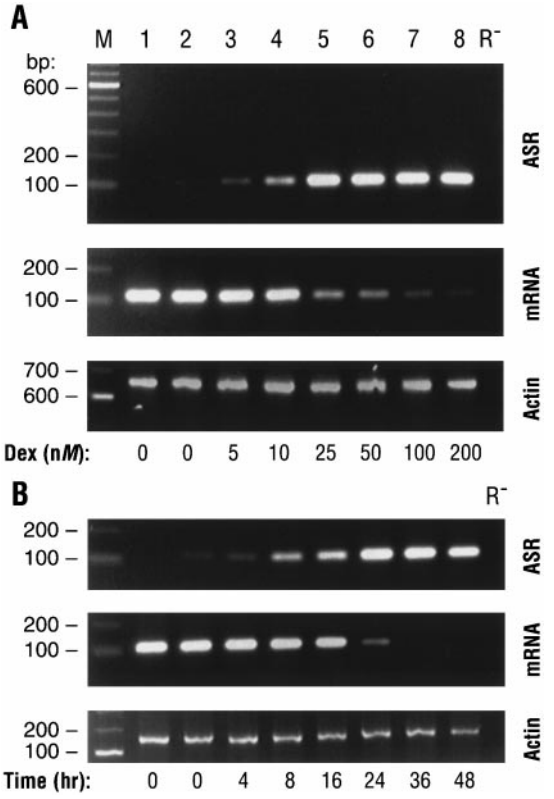

FIG. 3. (A) The expression pattern, as evaluated by RT-PCR, of *Wnt*-1 antisense RNA (ASR) in pGRE5-2/*tnW*-1 transfected R/Sa-MT cells (AS-13) and its effect on the blocking of *Wnt*-1 mRNA (mRNA) expression in response to a 24-h exposure to different concentrations (0–200 nM) of dexamethasone (Dex). (B) The time course of induction of *Wnt*-1 antisense RNA by 25 nM dexamethasone. M, molecular weight marker; lane 1 contained RT-PCR of control R/Sa-MT cell RNA. The lane designated R contained the RT-PCR product made from the RNA of R/Sa-MT cells in the absence of RT. The RT-PCR of actin mRNA (actin) was used as control.

To ascertain the responsiveness of antisense and sense constructs to dexamethasone, AS-8, AS-13, and AS-16 cell lines were treated with varying concentrations of the hormone, total cellular RNA was extracted, and the levels of the antisense and sense RNAs were evaluated by RT-PCR. We found that the AS-13 cell line expressed small amounts of antisense *Wnt*-1 RNA and that the levels of the RNA increased with hormonal treatment (Figs. 3A and 3B). Similar results were obtained with the AS-8 cell line (data not shown). In comparison with the control cells, the AS-13 cells treated with as little as 25 nM/ml (equivalent to 6.8 ng/ml) of dexamethasone for a period of 24 h resulted in an approximately 10-fold increase in the amount of antisense RNA (ASR; Fig. 3A, lane 5, and Fig. 3B, lane 6). The basal levels of endogenous *Wnt*-1 mRNA expression were found to be high in both the control R/Sa-MT (lane 1, Figs. 3A and 3B) and the transfected AS cells (lane 2, Figs. 3A and 3B). Treatment of the AS cells with increasing concentrations of the hormone resulted in increased expression of anti-

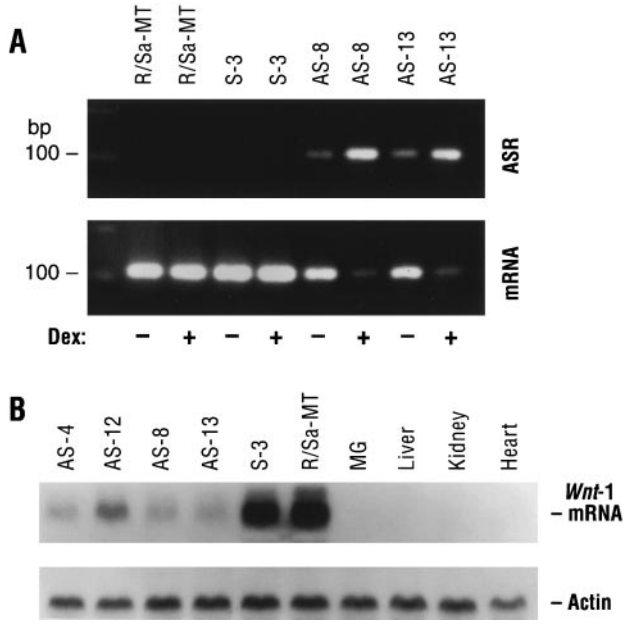

FIG. 4. (A) The effect, as determined by RT-PCR, of antisense *Wnt*-1 RNA (ASR) expression on the levels of the expression of *Wnt*-1 mRNA (mRNA) in two different antisense clones of R/Sa-MT cells (AS-8 and AS-13) grown to confluency in the presence (+Dex) or absence (−Dex) of 25 nM dexamethasone for a period of 24 h. R/Sa-MT cell RNA was used as a positive control, whereas the RNA from S-3 cells (sense clone) served as a negative control. (B) Northern blot hybridization with a *Wnt*-1 cDNA probe of RNA obtained from a number of dexamethasone (25 nM) treated R/Sa-MT cell lines containing sense (S-3) and antisense (AS-4, AS-8, AS-12, AS-13) constructs and from several mouse tissues (MG, mammary gland). Note that the presence of *Wnt*-1 mRNA is significantly lower in the antisense clones than in the R/Sa-MT (control) cells and cells that contained *Wnt*-1 sense constructs. Actin probe was used as a control to indicate uniform loading of RNA.

sense *Wnt*-1 RNA, and as a consequence the levels of the *Wnt*-1 mRNA decreased. The optimum concentration of the hormone was found to be 25 nM. It should be pointed out that this low concentration of hormone did not increase the levels of *Wnt*-1 mRNA despite the fact that the expression of the gene in these cells is under the regulation of an MMTV LTR that contains hormone responsive elements (data not shown). This is due to the fact that the MMTV LTR responds to a higher concentration (1–10 μg/ml, i.e., 3.7–37 mM) of dexamethasone (Ringold *et al.,* 1975; Sarkar *et al.,* 1977).

To define the optimum time of dexamethasone exposure for maximum induction of antisense RNA, the AS-13 cell line was treated with a 25 nM concentration of the hormone for various time periods and the levels of the expression of antisense RNA were evaluated by RT-PCR. The results indicated that the maximum amount of antisense RNA was induced with 20 to 30 h of hormonal exposure (Fig. 3B). The effects of dexamethasone treatment for a period of 24 h on the expression of *Wnt*-1 antisense and *Wnt*-1 mRNA in two clonal cell lines transfected with an antisense construct and in one cell line carrying a sense construct are shown in Fig. 4A. Compared to the control (RIII/Sa-MT) and the sense (S-3) cell lines, the hormone-treated antisense cell lines (AS-8 and AS-13) expressed approximately 7.8-fold more antisense RNA than the untreated AS-8 and AS-13 cells, and accordingly, the levels of endogenous *Wnt*-1 mRNA expression were dramatically reduced. These observations were confirmed further by Northern blot hybridization with a cDNA *Wnt*-1 probe of total RNA obtained from hormone-treated cells (Fig. 4B). The steady-state levels of *Wnt*-1 mRNA expression in the antisense cell lines AS-4, AS-12, AS-8, and AS-13 were found to be reduced by 91, 72, 89, and 84%, respectively, compared to the sense cell line S-3 or the control cell line. Similarly, the small tumors (see below) that were induced by the antisense cell lines expressed significantly much smaller amounts (80–90%) of *Wnt*-1 mRNA than the tumors produced by the sense or the control cell lines (data not shown).

The effect of antisense RNA on Wnt-1 protein expression

To determine whether the AS-8 and AS-13 cells carrying antisense constructs might fail to accumulate Wnt-1 protein, compared to cells carrying the sense constructs, cell extracts were prepared and subjected to immunoprecipitation with an appropriate anti-Wnt-1 peptide antiserum (see Materials and Methods). Quantitation of the immunoprecipitates following SDS–PAGE revealed that the amounts of Wnt-1 protein contained in the AS-8 and AS-13 cell lines were approximately 23.1 and 11.3%, respectively, of the amount of protein accumulated in the S-3 cell line (Fig. 5) or in the control cell line (data not shown). These observations are in close agreement with the levels of *Wnt*-1 mRNA expressed in these cell lines.

The effect of antisense RNA on cell growth and cell morphology

Assessment of the pattern of cell growth *in vitro* revealed that, in general, the antisense cell lines grew at a somewhat slower rate than the control (R/Sa-MT cell line) and the sense cell lines (Fig. 6). However, continuous dexamethasone (25 nM) treatment reduced significantly the growth rate of the antisense cell lines compared to the control and the sense cell lines. In the presence of the hormone, the antisense cell lines grew primarily in colonies and did not reach confluency. While the control and the sense cell lines with a seeding density of approximately $2 \times 10^6/75$ cm^2 required passaging within 5 days, the antisense cells remained stationary for up to 9 days with no appreciable cell loss.

The R/Sa-MT cells expressing *Wnt*-1 mRNA exhibited, prior to or at confluency, epithelioid morphology with elongated cytoplasm (Figs. 7A and 7E). Continuous ex-

posure of the cells to 25 nM dexamethasone did not affect the appearance of the cells (Figs. 7I and 7M). The morphology of the cells transfected with a *Wnt*-1 sense construct and grown in the absence (Figs. 7B and 7F) or in the presence (Figs. 7J and 7N) of dexamethasone resembled the nontransfected control cells (Figs. 7I and 7M). In contrast, the cells that were transfected with an antisense *Wnt*-1 construct displayed distinct morphological changes. In the absence of hormone, 1-day-old (Fig. 7C) or 2-day-old (Fig. 7G) cultures resembled the control cells, but with time some of the cells began to grow in distinct colonies (Figs. 7K and 7O). At confluency, about 20% of the cells exhibited this phenotype. Dexamethasone treatment of the cells showed profound changes in morphology; within 2–3 days postplating, the cells appeared to be organized into distinct foci or colonies of various sizes (Figs. 7H and 7L). Stationary cultures primarily showed the presence of cells with smaller amounts of cytoplasm (Fig. 7P); only a minor proportion of the cells (<5%) still showed extended cytoplasm. These observations clearly indicate that *Wnt*-1 antisense RNA expression profoundly affected the morphology of the *Wnt*-1 RNA-expressing R/Sa-MT cells and that this effect seems to be directly related to the antisense downregulation of the levels of endogenous *Wnt*-1 mRNA and protein.

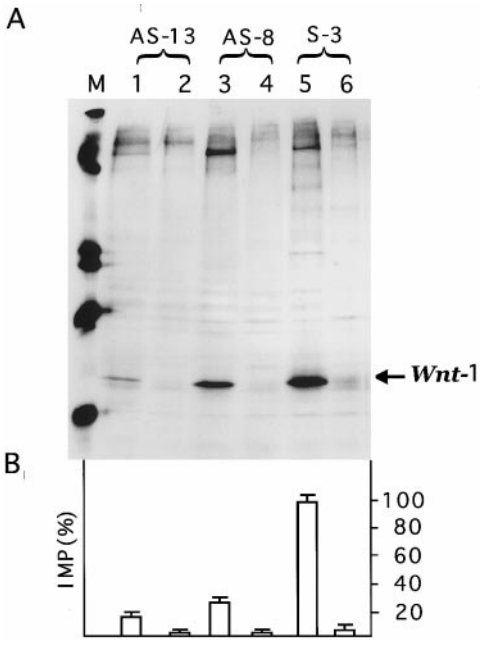

FIG. 5. Comparison of Wnt-1 protein levels in mouse mammary tumor cells transfected with antisense (AS-13 and AS-8) and sense (S-3) *Wnt*-1 constructs. Cells were labeled with [^{35}S]cysteine, cell extracts were prepared, and samples of each cell extract containing equivalent amounts of ^{35}S counts/minute were immunoprecipitated with 2 μl JP4 antiserum (A, lanes 1, 3, and 5). Cell extracts were also immunoprecipitated with serum preabsorbed with synthetic Wnt-1 peptide (lanes 2, 4, and 6). (B) The relative amounts of Wnt-1 protein that were available for immunoprecipitation (IMP) from each of the three cell lines. An arbitrary unit of 100 was assigned to the density of the protein band corresponding to the sense cell line S-3.

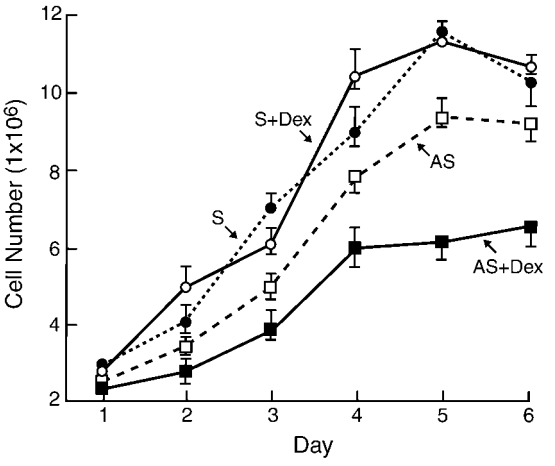

FIG. 6. *In vitro* growth patterns of sense (S-3) and antisense (AS-13) cell lines unexposed (S and AS) or exposed (S + Dex, AS + Dex) continuously to 25 nM dexamethasone (Dex). Triplicate cultures were used for each time point. Each culture dish (75 cm^2) was seeded with 2×10^6 cells.

The effect of *Wnt*-1 antisense RNA expression on the tumorigenicity of R/Sa-MT cells

To evaluate the biological consequences of the downregulation of *Wnt*-1 mRNA and protein in cells containing antisense *Wnt*-1 constructs, we tested the tumorigenicity of the antisense cell lines AS-8 and AS-13 in RIII/Sa mice. R/Sa-MT cells transfected with sense constructs (S-3) and nontransfected cells were also used as controls. Representative data from these experiments are shown in Table 1. About 10% of the mice inoculated with R/Sa-MT cells, irrespective of dexamethasone treatment, developed palpable tumors within a week, and all mice showed palpable tumors by 3 weeks. Growth patterns of the tumors from cells transfected with sense constructs and from control cells were found to be very similar. By contrast, most of the mice inoculated with cells expressing antisense RNA did not show palpable tumors until 3–4 weeks postinoculation. Dexamethasone treatment of these animals appeared to cause some delay in the appearance of the tumors. For example, it took approximately 9 weeks for all of the hormone-treated animals to develop tumors compared to a period of 6 weeks for the untreated mice.

The profound inhibitory effect of antisense RNA on tumor growth became apparent when the average sizes of the tumors extracted from the different groups of mice were compared (Table 1). Mice transplanted with antisense RNA expressing cells and treated with dexamethasone produced tumors no larger than 0.3 cm in diameter, even after a period of 16 weeks (Fig. 8B). Under identical conditions, the control groups of mice produced

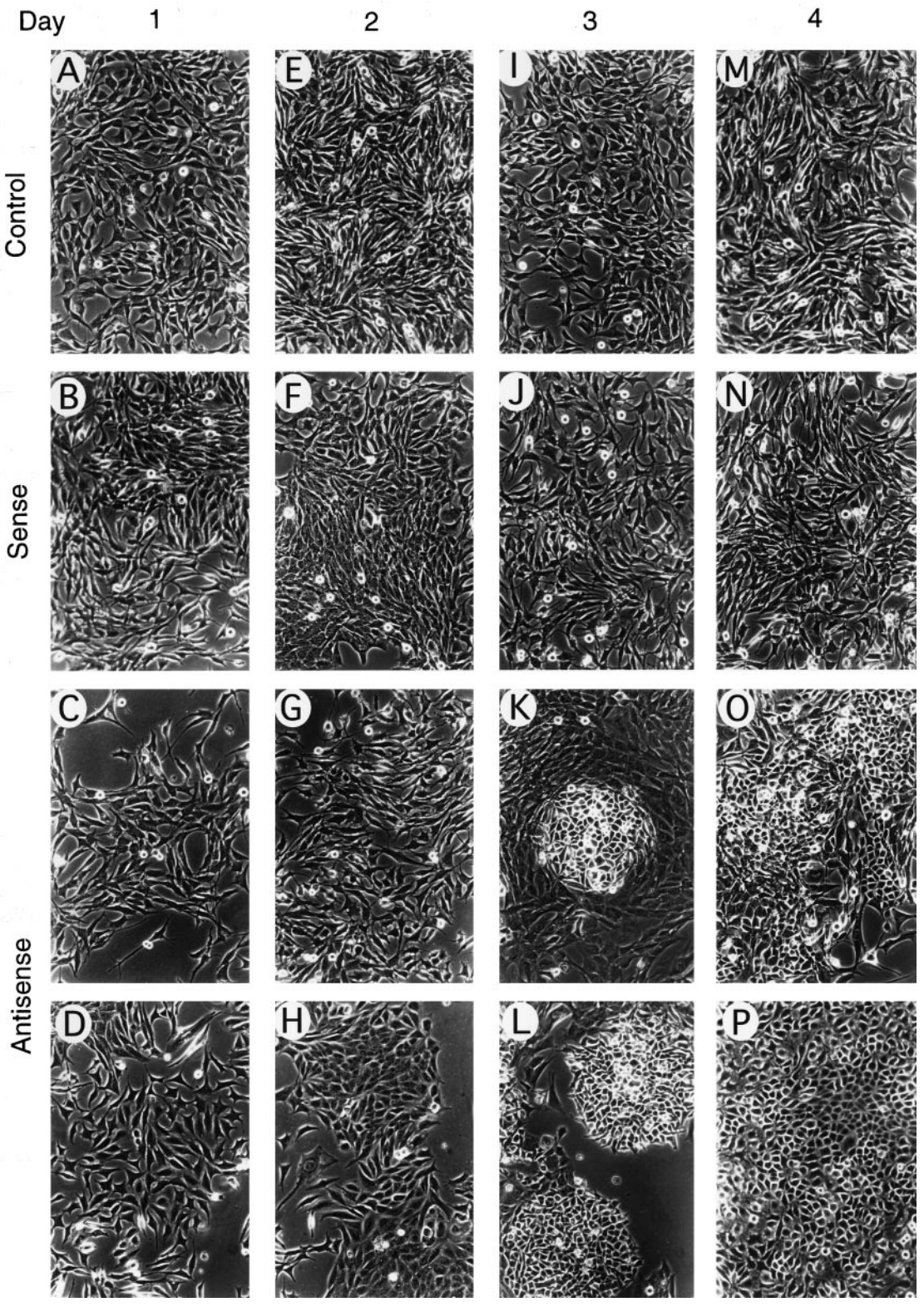

FIG. 7. The morphology of control (nontransfected) R/Sa-MT cells (A, E, I, and M) and of cells containing sense (B, F, J, and N) and antisense (C, D, G, H, K, L, O, and P) *Wnt*-1 constructs as observed after 1 (A–D), 2 (E–H), 3 (I–L), and 4 (M–P) days postplating of 3×10^6 cells in a 75-cm^2 dish. Some of the cultures were treated continuously with 25 nM dexamethasone (D, H, I, J, L, M, N, and P). Note that the cells that were expressing low levels of antisense transcripts (in the absense of dexamethasone) exhibited some alterations in morphology as the cultures became confluent (K and O). Such a change in cell morphology became more pronounced with increased expression of the *Wnt*-1 antisense RNA as a consequence of dexamethasone treatment (L and P). It should be noted that P represents the appearance of a large colony of cells.

TABLE 1

The Effect of Sense and Antisense *Wnt*-1 RNA Expression on the Tumorigenicity of R/Sa-MT Cells

Cells transfected with or without *Wnt*-1 constructs	No. of mice inoculated (Dex ±)[a]	Time for the appearance of tumor (weeks)[b]			Tumor load	
		T_a	T_{50}	T_{100}	Time of sacrifice (weeks)[c]	Tumor size (cm)
R/Sa-MT + none	16 (Dex−)	1	1.5	3	8	$2.0 \pm 0.3^{\alpha}$
R/Sa-MT + none	16 (Dex+)	1	1.5	3	8	$2.1 \pm 0.3^{\alpha}$
R/Sa-MT + sense (cl. S-3)	21 (Dex−)	1	2.0	4	8	$2.1 \pm 0.4^{\alpha}$
R/Sa-MT + sense (cl. S-3)	18 (Dex+)	1	2.0	4	8	$2.1 \pm 0.4^{\alpha}$
R/Sa-MT + antisense (cl. As-8)	26 (Dex−)	3	5.0	8	12	$1.9 \pm 0.2^{\alpha}$
R/Sa-MT + antisense (cl. As-8)	25 (Dex+)	4	7.0	9	16	$0.3 \pm 0.1^{\beta}$
R/Sa-MT + antisense (cl. As-13)	13 (Dex−)	3	5.0	6	12	$1.5 \pm 0.2^{\alpha}$
R/Sa-MT + antisense (cl. As-13)	15 (Dex+)	4	6.0	9	16	$0.2 \pm 0.1^{\beta}$

[a] Mice were treated with (Dex +) or without (Dex −) dexamethasone (10 ng/g of body weight/day).

[b] T_a, time (weeks) postinoculation for the earliest appearance of palpable tumors in each group of mice; T_{50}, the time when 50% of the mice developed tumors; T_{100}, the time when 100% of the mice had developed tumors.

[c] Animals were sacrificed at the end of 8, 12, or 16 weeks postinoculation. The different time periods were chosen because of the fact that tumor bearing animals had to be sacrificed as soon as tumor size reached approximately 2.0 cm in diameter. Tumor sizes noted by dissimilar superscripts differ significantly ($P < 0.001$).

large tumors, 1.8 to 2.4 cm in diameter, within 8 weeks (Fig. 8A). It is of interest to note that these tumors grew very rapidly and were often found to be composed of multiple segments.

DISCUSSION

We have demonstrated in the present study that the ability of a tumor cell line (R/Sa-MT) to form tumors *in vivo* can be modulated by transfecting the cells with a plasmid construct containing *Wnt*-1 sequences in the antisense orientation. The R/Sa-MT cell line expresses *Wnt*-1 mRNA and protein and produces large tumors (≃2.0 cm in diameter) in syngeneic mice within a period of 8 weeks (Table 1). Compared to these cells, or to cells that were transfected with a plasmid DNA containing *Wnt*-1 sequences in the sense orientation, the antisense expressing cells (R/Sa-MT/antisense) took twice the amount of time to form palpable tumors (6–8 weeks vs 3–4 weeks) and the tumors they formed were 80–90% smaller in diameter. Our analyses by RT-PCR of the expression of *Wnt*-1 antisense RNA and *Wnt*-1 mRNA in the R/Sa-MT/antisense cells were not quantitative; however, qualitatively, the levels of the respective RNAs were found to be inversely proportional (see Figs. 3A, 3B, and 4A). Furthermore, the RT-PCR results correlated very closely with those obtained by Northern hybridizations (Fig. 4B). These observations, therefore, suggest that the primary effect of antisense *Wnt*-1 expression is the downregulation of *Wnt*-1 mRNA; the antisense RNA most likely blocked *Wnt*-1 transcription as a result of its interaction with the sense strand DNA. The possibility remains that the antisense RNA also may have blocked the transfer of *Wnt*-1 pre-mRNA to the cytoplasm. It should be mentioned that currently, the mechanism of action of an antisense gene is not well understood, but it has been speculated that antisense expression may interfere with the functioning of a gene at the level of RNA transcription, processing, transport, translation, or mRNA stability (Izant, 1992).

The presence of smaller amounts of *Wnt*-1 protein in the antisense cell lines, compared to the normal and sense cell lines (Figs. 5A and 5B), seems to account for the loss of the highly tumorigenic phenotype of the R/Sa-MT cells (Table 1). In general, we observed close correspondence between the copy number of the integrated antisense construct contained in different antisense cell lines, the levels of antisense RNA expression, the presence of *Wnt*-1 mRNA and protein in the corresponding cell lines, and the efficiency of the clones to form tumors *in vivo*. However, the relationship between the extent of antisense downregulation of *Wnt*-1 mRNA/protein and the growth rate of the antisense cells *in vivo* was found to be nonlinear. For example, the antisense clone 13 contained more copies (seven copies) of the antisense DNA than the antisense clone 8 (three copies) and expressed significantly smaller amounts of *Wnt*-1

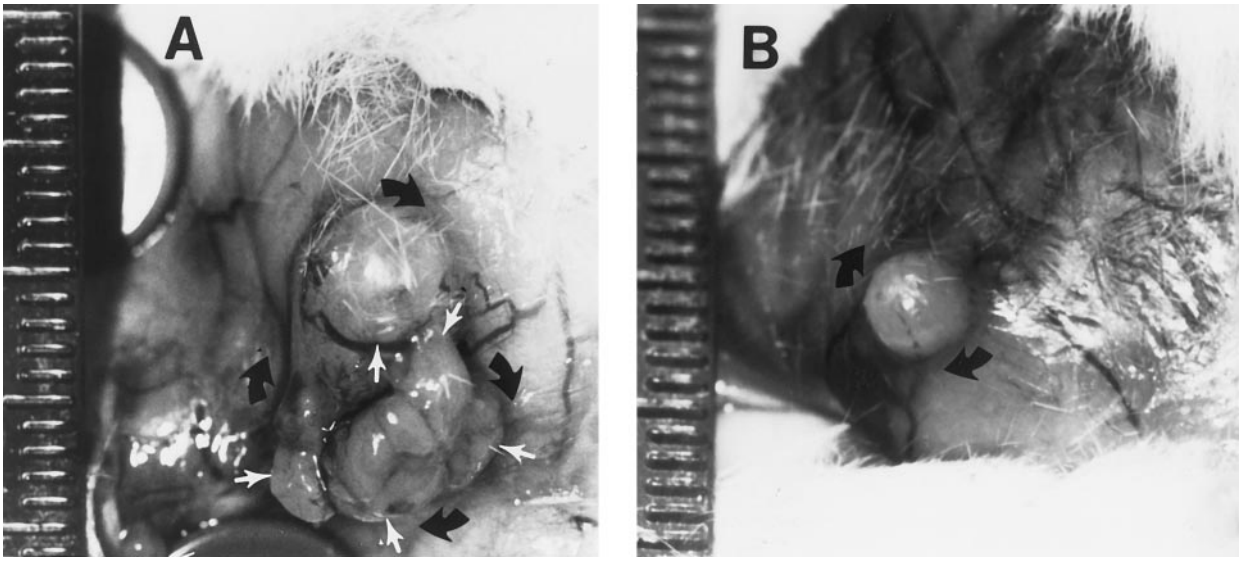

FIG. 8. The appearance of mammary tumors produced by control R/Sa-MT cells (A) and cells expressing *Wnt*-1 antisense transcript (B). Each mouse was injected with 5×10^6 cells subcutaneously on the same day, and from the third day on, each mouse was injected daily with dexamethasone (25 nM/mouse). The mouse in A was sacrificed after 8 weeks, while the mouse in B was sacrificed after 16 weeks. A and B represent the smallest and the largest of the tumors that developed in the two respective groups of animals. The black curved arrows outline the tumors; the small white arrows point to individual tumor lobes.

mRNA/protein (Figs. 4B and 5) than clone 8, yet both cell lines produced tumors of similar sizes (Table 1). These observations indicate that there may be a threshold between the level of *Wnt*-1 mRNA/protein expression and the ability of the cells to develop tumors. This notion may provide an explanation, at least in part, for the variability observed by many investigators in the levels of expression of *Wnt*-1 mRNA and other *int* protooncogenes, such as *Fgf*-3 (*int*-2) and *int*-3, among most, if not all, spontaneously developed mammary tumors of mice (for references see Sarkar, 1995). The other factor that may account for these variations is the polyclonality of individual tumors with respect to the activation of the *int* genes (Sarkar, 1995).

Inappropriate expression of *Wnt*-1 has been attributed to the transformation of normal mammary epithelial cells into preneoplastic and neoplastic cells. *Wnt*-1 also seems to play an important role in the development of mouse as well as *Xenopus* embryos (McMahon and Bradley, 1992; McMahon and Moon, 1989). Since coordinated cell proliferation and maintenance of close association between groups of cells via cell–cell adhesion are essential in development (Gerhart and Keller, 1986), it is reasonable to assume that *Wnt*-1 may act *in vivo* not only as a regulator of cell proliferation and differentiation, but also as a modulator of cellular contacts. The consequences of ectopic *Wnt*-1 expression in a number of cell lines have been examined (Brown *et al.,* 1986; Rijsewijk *et al.,* 1987; Jue *et al.,* 1992). *Wnt*-1 expression was found to alter the cell morphology of one normal mammary epithelial cell line (C57BL) from a flat, cuboidal type to elongated, refractile, fibroblast-like cells (Brown *et al.,* 1986; Jue *et al.,* 1992). The cells, although not fully transformed, showed another phenotypic change: unlike the control cells, they acquired the ability to divide in culture beyond confluency and to exhibit increased calcium-dependent cell–cell adhesion (Brown *et al.,* 1986; Jue *et al.,* 1992; Hinck *et al.,* 1994b).

Ectopic *Wnt*-1 expression in a mammary tumor cell line that had a MMTV integration at the *Fgf*-3 locus, but not at the *Wnt*-1 locus, showed some changes in cell growth and morphology; the cells grew to a higher density, they showed disordered, criss-cross patterns, and their shape changed from a cuboidal to an elongated type (Rijsewijk *et al.,* 1987). Interestingly, *Wnt*-1 expression has also been observed to cause morphological changes in one nonmammary, neural crest-derived tumor cell line PC12 (Bradley *et al.,* 1993; Shackleford *et al.,* 1993b). Unlike the control cells, the *Wnt*-1 expressing PC12 cells (PC12/*Wnt*-1) were found to grow in discrete colonies of adherent cells with an epitheloid appearance. Similarly, as shown in the present study, antisense downregulation of *Wnt*-1 seemed to induce significant phenotypic changes in the morphology of the R/Sa-MT/antisense cells *in vitro.* The *Wnt*-1 expressing cells (R/Sa-MT) appeared to be elongated and disordered, whereas the cells in which endogenous *Wnt*-1 expression was blocked exhibited cuboidal morphology (Fig. 7). The most important aspect of our finding is the causal association between the levels of *Wnt*-1 mRNA/protein expression, morphological changes, and tumorigenicity of the cell lines. However, the signal transduction pathway of *Wnt*-1 in our cell line remains unknown.

There are strong indications that the protein encoded

by *Wnt*-1 acts as an intracellular signaling molecule, as do the transforming growth factors (*TGF*s) (Massague, 1992; Moses, 1990) and fibroblast growth factors (*FGF*s) (Burgess and Maciag, 1989; Klagsburn, 1989; Rifkin and Moscatelli, 1989). A number of studies have shown that ectopic expression of *Wnt*-1 results in the accumulation of plakoglobin and/or β-catenin in some cells (Bradley *et al.,* 1993; Funayama *et al.,* 1995; Hinck *et al.,* 1994a, b; Papkoff *et al.,* 1996). Furthermore, *Wnt*-1 expressing PC12 cells show increased levels of plakoglobin and E-cadherin and increased levels of calcium-dependent cell–cell adhesion (Bradley *et al.,* 1993). These findings suggest that β-catenin and plakoglobin may be the targets of the *Wnt*-1 signal transduction cascade. Since β-catenin and plakoglobin bind to the cytoplasmic domain of the cadherin family of cell adhesion molecules (Nagafuchi and Takeichi, 1988; Knudsen and Wheelock, 1992; Piepenhagen and Nelson, 1993), it is likely that *Wnt*-1 functions through β-catenin and plakoglobin in eliciting morphogenetic changes in cells. In this pathway, the involvement of glycogen synthase kinase 3β (GSK3β) and the T cell factor/lymphocyte enhancer binding factor has been suggested (Cook *et al.,* 1996; Behrens *et al.,* 1996; Porfiri *et al.,* 1997; Papkoff and Aikawa, 1998; Nusse, 1997). *Wnt*-1-induced cell fate determination may also involve the participation of *Engrailed*-1 (Danielian and McMahon, 1996) and the gap-junctional protein connexin 43 (van der Heyden *et al.,* 1998).

At present, it is virtually unknown whether or not changes in β-catenin/plakoglobin and/or connexin 43 expression may affect the growth potential of *Wnt*-1-induced mammary tumor cells *in vivo.* In this context, it should be noted that abnormal expression of E-cadherin, a modulator of the cytoplasmic pools of β-catenin, has been found to occur frequently in adenocarcinomas such as breast cancer, gastric cancer, and prostate cancer (see Voeller *et al.,* 1998). As shown in the present study, our R/Sa-MT cell line is highly tumorigenic, and therefore, it could be used as an ideal model system for the identification of the downstream molecules that are involved in the *Wnt*-1 signal transduction pathway during tumorigenesis of the mouse mammary glands. We are currently interested in determining whether or not antisense downregulation of β-catenin/plakoglobin and/or connexin 43 will modulate the tumorigenic phenotype of this cell line in a manner similar to what we have observed with the downregulation of *Wnt*-1.

MATERIALS AND METHODS

Plasmid construction

The coding region of the *Wnt*-1 gene is composed of four exons with a single open reading frame that encodes a protein of 370 amino acids (Fung *et al.,* 1985). The mode of action of antisense RNA-mediated inhibition of gene expression presumably involves hybridization of antisense RNA to a target mRNA (Freir *et al.,* 1992). It is therefore desirable that the sequences targeted for the antisense RNA do not contain stem loop structures containing more than 6-bp stems. On the basis of the secondary structural analysis of *Wnt*-1 cDNA (using the CCG Program from Genetic Computer Group, Inc.), we selected a 290-bp segment of *Wnt*-1 that contained the initiation codon for Wnt-1 protein (Fung *et al.,* 1985). This segment of *Wnt*-1 was released by digestion with *Eco*RI and *Xho*I from pSP65 plasmid DNA that contained a copy of full-length *Wnt*-1 cDNA (Fig. 1). The digested DNA was subjected to preparative electrophoresis in a 1.2% agarose gel and the appropriate fragment was isolated by electroelution and ligated separately into the eukaryotic expression vectors pGRE5-1 and pGRE5-2 (kindly provided by Sylvie Mader of McGill University, Canada; Mader and White, 1993) at the *Eco*RI/*Xho*I and *Xho*I/*Eco*RI cloning sites to create *Wnt*-1 sense (pGRE5-1/*Wnt*-1) and antisense (pGRE/*tnW*-1) constructs, respectively.

Tissue culture and cell transfection

The mammary tumor cell line (R/Sa-MT) used in this study was established in our laboratory from a lung nodule that developed in an RIII/Sa mouse carrying a transplantable metastatic mammary tumor. This epithelial cell line expresses constitutively high levels of *Wnt*-1 mRNA and protein as a consequence of insertional mutation of the oncogene by a MMTV provirus. Transplantation of R/Sa-MT cells in syngeneic male or female mice results in rapid tumor development. A full characterization of this cell line will be reported elsewhere. Routine cell cultures were done in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, nonessential amino acids, L-glutamine, vitamins, and antibiotics. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

The pGRE5-1/*Wnt*-1 and pGRE5-2/*tnW*-1 plasmid DNAs, carrying the sense and antisense *Wnt*-1, respectively, were cotransfected with pSV_2neo (Southern and Berg, 1982) plasmid DNA into the R/Sa-MT cells using the standard calcium phosphate ($CaPO_4$) transfection procedure (Chen and Okayama, 1988). A total of 30 μg DNA at a 20:1 molar ratio of sense/antisense to pSV_2neo DNA was used for each transfection. The transfected cells were then cultured from day 2 onward in the same medium, but supplemented with 400 mg/ml of G418 (Geneticin; Gibco Laboratories, Grand Island, NY) in order to select stable transfectants. After 12–14 days, G418-resistant colonies were isolated and replated in fresh medium for the expansion of the clones.

Determination of cell growth

Equal numbers of cells (1 or 2 × 10^6/75-cm^2 dishes) from the parental, antisense, and sense cell lines were

seeded and cultured for various days in the presence or in the absence of dexamethasone (25 nM). Cells were harvested by trypsinization, resuspended in isotonic diluent, and counted with a hemocytometer.

Inoculation of mice

To compare the tumorigenicity of the R/Sa-MT cells transfected with sense or antisense *Wnt*-1 constructs, as well as control cells, eight groups of 4- to 6-week-old RIII/Sa (Sarkar, 1995) mice were inoculated. Monolayer cultures were trypsinized with 0.05% trypsin in 0.01% EDTA and the cells were counted with a hemocytometer. Approximately 5×10^6 cells suspended in a minimum volume (100–200 μl) of ice-cold PBS were injected into the fourth inguinal mammary fat pads of mice. The inoculated mice were maintained under identical conditions with respect to environment, diet, and handling except that four groups of mice were inoculated daily with 20–25 μl of solution containing predetermined amounts (10 ng per gram of body weight) of dexamethasone while the control groups were inoculated with solvent. The fat pads of each mouse were palpated daily from the fifth day postinoculation for the appearance of tumors. The mice continued to be under daily observation for a period of 16 weeks and the approximate size of tumors was estimated on a weekly basis with a Vernier caliper. During this time period, those mice that had tumors close to 2.0 cm in diameter were sacrificed, their tumors were excised, and the size of the tumors was determined.

Southern analysis

Ten micrograms of DNA isolated from each of the clonal cell lines was digested to completion using *Eco*RI under conditions recommended by the manufacturer (New England Biolabs Inc., Beverly, MA). Digested DNA was electrophoresed in a 0.8% Tris–acetate agarose gel and transferred to nylon filter (Hybond-N; Amersham Corp., Arlington Heights, IL). The filter was hybridized with a [^{32}P]dCTP-labeled *Wnt*-1 cDNA probe, washed, and exposed to Kodak XAR film following the procedure that we have used previously (Sarkar, 1995).

Northern hybridization

Total cellular RNA was extracted from exponentially growing cells with 6 M urea–3 M LiCl, denatured, and electrophoresed in 1% agarose gel (20 μg RNA/lane) according to the procedure that we have described previously (Li *et al.,* 1994). RNA was transferred to Zeta Probe filters in the presence of 20× SSC (1× SSC consists of 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and UV cross-linked. The filters were hybridized with approximately 5×10^7 cpm/ml of radioactive probes in a mixture containing 0.2 M Na_2HPO_4, 1 mM EDTA, 1% BSA, 7% SDS, and 16% formamide for 24 h at 65°C. The filters were then washed three times, 25 min each, in a solution of 0.04 M Na_2HPO_4, 5% SDS, and 0.001 mM EDTA at 65°C and exposed to Kodak X-ray film at −80°C. The radioactive probe was then stripped (dehybridized) from the filter and the filter was rehybridized with a chicken β-actin probe in order to ensure that equivalent amounts of RNA were loaded on each lane of the gel.

Reverse transcriptase and RT-PCR

Two micrograms of total cellular RNA was digested with 2 U of RQ1 RNase-free DNase (Promega Corp., Madison, WI) in the presence of 20 U of RNasin (Promega Corp.) in 10 μl at 37°C for 15 min followed by DNase inactivation at 95°C for 2 min. To detect the expression of *Wnt*-1 antisense RNA, the sense primer (forward) was used in a reverse transcription reaction, while the antisense primer (reverse) was used to detect cellular *Wnt*-1 mRNA. The reverse transcriptase reaction was performed with 5 U of AMV reverse transcriptase (Promega) in 50 mM Tris–Cl, pH 8.3, 50 mM KCl, 10 mM DTT, 5 mM $MgCl_2$, 0.5 mM spermidine, 20 U RNasin, 0.2 mM each dNTP, and 10 pmol of antisense or sense primers for 60 min at 42°C in a final volume of 20 μl. Of the resulting cDNA, 4 μl was used for a PCR containing 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris–Cl (pH 9.0), 0.1% Triton X-100, a 0.2 mM concentration of each dNTP, 16 pmol (100 ng) of each of the *Wnt*-1 or β-actin primers, and 1 U of *Taq* polymerase (Promega) at a final volume of 50 μl.

Amplification of *Wnt*-1 cDNA was done with a GeneAmp PCR System 9600 (Perkin–Elmer, Foster City, CA) for 27 cycles according to the following program: 20 s at 95°C, 20 s at 65°C, and 15 s at 72°C. The program for the amplification of β-actin was 27 cycles of 95°C for 20 s, 62°C for 20 s, and 72°C for 35 s. Samples were analyzed by electrophoresing 25 μl of each product in 2% agarose gels and staining with ethidium bromide. The following primers were used for RT-PCR: for *Wnt*-1 cDNA, the forward primer sequence was 5′(204)-GCCCAGCTGGGTTTCTACTAC(224)-3′ (also used for reverse transcription of *Wnt*-1 antisense RNA), and the reverse primer sequence was 5′(311)-AGGAGGCTATGTTCACGATGC-(290)-3′ (also used for cellular *Wnt*-1 mRNA reverse transcription). The expected size of the amplified *Wnt*-1 cDNA product from reverse transcription was 107 bp. The primers for β-actin were forward primer sequence, 5′ (389)-TGACGGGGTCACCCACACTGTGCCCATCTA(419)-3′; reverse primer sequence, 5′ (1050)-CTAGAAGCACTTGCGGTGCACGATGGAGGG(1021)-3′. The size of the β-actin product was expected to be 661 bp (GenBank Accession No. 03765).

Immunoprecipitation

Metabolic labeling of cells and immunoprecipitation of Wnt-1 protein were done as previously described (Papkoff *et al.,* 1987; Bradley and Brown, 1990; Papkoff and

Schryver, 1990). Briefly, cells were grown to subconfluency (60-mm dish) in DMEM containing 20 nM/ml of dexamethasone, washed with phosphate-buffered saline (PBS), and incubated for 30 min in cysteine-free DMEM. The dishes were then replenished with fresh medium containing 500 μCi of [^{35}S]cysteine (Amersham Corp.), incubated for 2 h, washed, and reincubated in the presence of dexamethasone for an additional period of 4 h. After two rinses in PBS, the cells were lysed at 4°C in 1 ml of RIPA buffer composed of 0.01 M sodium phosphate, 0.15 M NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% aprotinin. The cell extracts were centrifuged in a microfuge for 15 min at 4°C, and the supernatants were immunoprecipitated in a volume of 300 μl for 2 h on ice with 2 μl of JP4 antiserum directed against *Wnt*-1 peptide residues 275 to 289 (Papkoff *et al.,* 1987).

Immunoprecipitation of cell extracts was also done with JP4 antiserum that was preincubated with the appropriate peptide (1 μg/1 μl serum) for 30 min. Following immunoprecipitation, 1 mg of protein A–Sepharose (Boehringer Mannheim Biochemicals) was then added to each sample and the reaction mixtures were mixed periodically at 4°C for 45 min. The protein A–Sepharose–antibody complexes were pelleted through 300 μl of RIPA buffer plus 10% sucrose in a microfuge and washed once with the RIPA buffer. The pellets were then suspended in 2× sample buffer (125 mM Tris–hydrochloride, 4% SDS, 10% β-mercaptoethanol, 10% glycerol), boiled for 2 min, and loaded onto SDS–15% polyacrylamide gels. After electrophoresis, the gels were fixed in 10% acetic acid–25% methanol, treated with Amplify (Amersham), dried, and fluorographed. The intensity of the radioactive signals was quantified using a Molecular Analyst software program (Bio-Rad Laboratories).

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